




ORIGINAL ARTICLE

In vitro data suggest that Indian delta variant B.1.617 of SARS-CoV-2 escapes neutralization by both receptor affinity and immune evasion

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Abstract

Background: Emerged mutations can be attributed to increased transmissibility of the B.1.617 and B.1.36 Indian delta variants of SARS-CoV-2, most notably substitutions L452R/E484Q and N440K, respectively, which occur in the receptor-binding domain (RBD) of the Spike (S) fusion glycoprotein.

Objective: We aimed to assess the effects of mutations L452R/E484Q and N440K (as well as the previously studied mutation E484K present in variants B.1.351 and P.1) on the affinity of RBD for ACE2, SARS-CoV-2 main receptor. We also aimed to assess the ability of antibodies induced by natural infection or by immunization with BNT162b2 mRNA vaccine to recognize the mutated versions of the RBD, as well as blocking the interaction RBD-ACE2, an important surrogate readout for virus neutralization.

Methods: To this end, we produced recombinant wild-type RBD, as well as RBD containing each of the mutations L452R/E484Q, N440K, or E484K (the latest present in variants of concern B.1.351 and P.1), as well as the ectodomain of ACE2. Using Biolayer Interferometry (BLI), we measured the binding affinity of RBD for ACE2 and the ability of sera from COVID-19 convalescent donors or subjects immunized with BNT162b2 mRNA vaccine to block this interaction. Finally, we correlated these results with total anti-RBD IgG titers measured from the same sera by direct ELISA.

Results: The binding assays showed L452R/E484Q double-mutant RBD to interact with ACE2 with higher affinity ($K_D = 4.6$ nM) than wild-type ($K_D = 21.3$ nM) or single mutants N440K ($K_D = 9.9$ nM) and E484K ($K_D = 19.7$ nM) RBDs. Meanwhile, the anti-RBD IgG titration resulted in lower recognition of mutants E484K and L452R/E484Q by infection-induced antibodies, whereas only mutant E484K was recognized less by antibodies induced by vaccination. More interestingly, sera from convalescent as well

Abbreviations: ACE2, angiotensin-converting enzyme 2; BLI, biolayer interferometry; COVID-19, coronavirus disease 2019; RBD, receptor-binding domain; RBD_{E484K}, RBD containing mutation E484K on S; RBD_{L452R/E484Q}, RBD containing both mutations L452K and E484Q on S; RBD_{N440K}, RBD containing mutation N440K on S; RBD_{WT}, wild-type RBD; RBM, receptor-binding motif; S, SARS-CoV-2 spike glycoprotein; SARS-CoV-2, severe acute respiratory syndrome coronavirus type 2.

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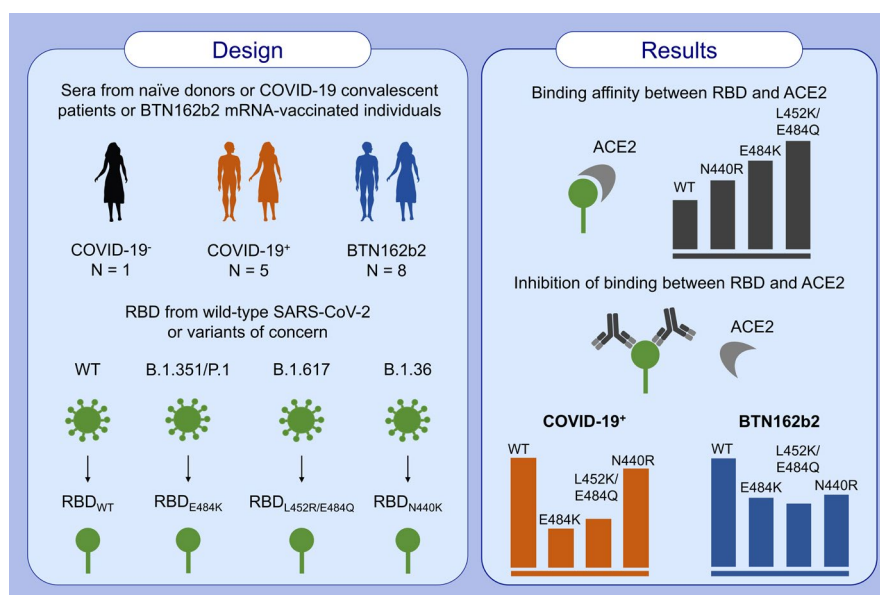
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as immunized subjects showed reduced ability to block the interaction between ACE2 and RBD mutants E484K and L452R/E484Q, as shown by the inhibition assays.

Conclusion: Our data suggest that the newly emerged SARS-CoV-2 variant B.1.617, as well as the better-studied variants B.1.351 and P.1 (all containing a mutation at position E484) display increased transmissibility both due to their higher affinity for the cell receptor ACE2 and their ability to partially bypass immunity generated against the wild-type virus. For variant B.1.36 (with a point mutation at position N440), only increased affinity seems to play a role.

KEYWORDS

affinity, neutralization, RBD, SARS-CoV-2, vaccine



GRAPHICAL ABSTRACT

Binding assays using Biolayer Interferometry showed that the RBD containing L452R/E484Q present in the new SARS-CoV-2 variant B.1.617 interacts with ACE2 with higher affinity than wild-type or single mutants N440K and E484K. Sera from convalescent and BTN162b2 vaccinated individual showed reduced ability to block the interaction between ACE2 and RBD mutants E484K and L452R/E484Q suggesting that the new SARS-CoV-2 variant B.1.617 bypass immunity generated against the wild-type virus.

1 | INTRODUCTION

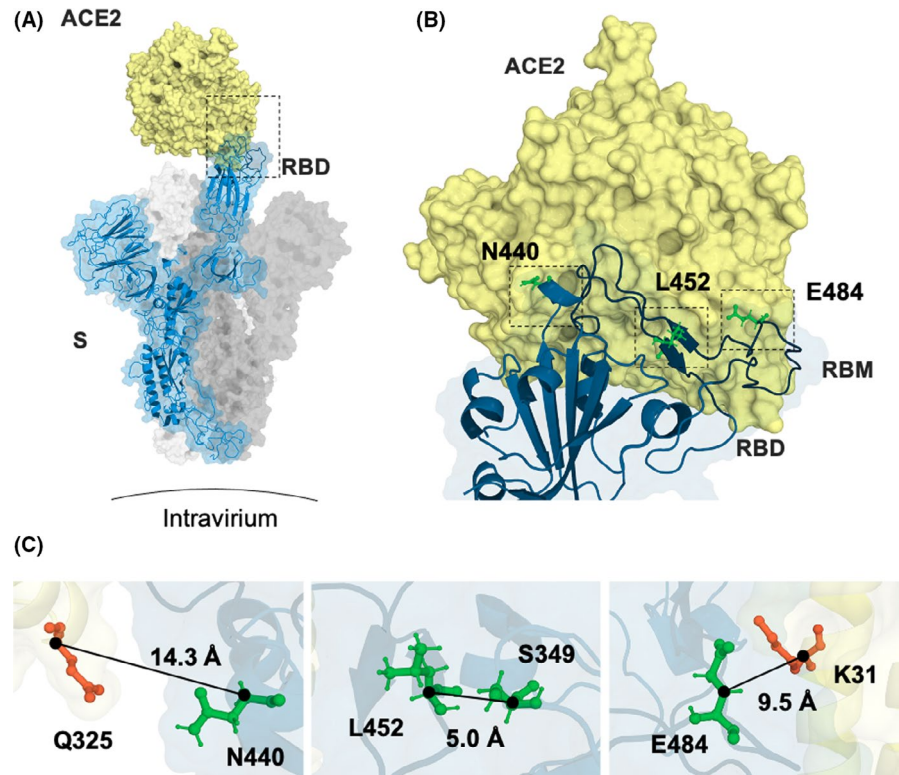
Antibodies, in particular neutralizing antibodies, are the primary effector mechanism induced by anti-viral vaccines. Hence, for vaccines against COVID-19, neutralizing antibodies are expected to be the correlate of protection.^{1,2} It is known that for SARS-CoV-2, the receptor-binding domain (RBD) of the Spike fusion glycoprotein is the primary target for neutralizing antibodies, and that ELISA antibody titers against the RBD tightly correlate with virus neutralization.³

The receptor-binding motif (RBM) is the segment of the RBD in closest contact with the host receptor angiotensin-converting enzyme 2 (ACE2, Figure 1A/B), and, because of its relevance for the virus attachment, it is the most important binding site for

neutralizing antibodies.⁴ The importance of RBD/RBM is underlined by the observation that prominent variants of concern (e.g., South Africa B.1.351 and Brazil P.1) show strongly reduced susceptibility to neutralizing antibodies due to equally reduced recognition by serum antibodies caused by E484K mutation located in the RBM.^{5,6} In contrast, variants lacking the E484K mutation (e.g., UK B.1.1.7) are well recognized by convalescent and immunized sera, with the RBD-ACE2 interaction barely affected by these antibodies.⁷ It seems though that a slight increase in the affinity of the RBD for the receptor (1.6-fold) can lead to increased viral infectivity.

Here, we show that the Indian delta variant B.1.617 of SARS-CoV-2 has acquired higher infectivity and transmissibility (therefore becoming the dominant strain in many countries) by both enhanced affinity for ACE2 and neutralizing immune evasion. In variant B.1.36,

FIGURE 1 Positions of mutations N440K, L452R, and E484Q. (A) S monomer (blue ribbon and surface) bound to ACE2 ectodomain (yellow surface). (B) This interaction is shown in detail, highlighting residues N440, L452, and E484 (green sticks). Interestingly, both residues L452 and E484 are found in close contact with ACE (ie, at the RBM), whereas residue N440 is relatively distant from the interaction surface (ie, out of the RBM). (C) Distance from the nearest residue for N440, L452, and E484 (residues from the RBD shown in green and from ACE2 in orange). From PDB files 6ACG and 2AJF



on the other hand, only increased affinity for ACE2 plays a role, making this variant more similar to the UK B.1.1.7.

2 | MATERIAL AND METHODS

2.1 | Gene cloning

The constructs for RBD_{WT} and mutants E484K, L452R/E484Q, and N440K were all designed as single genes containing each protein of interest fused to an N-terminal μ -phosphatase signal peptide and a C-terminal hexahistidine tag. An Avitag™ BAP sequence followed by an EPEA C-tag replaced the 6xHis tag in the ACE2-expressing construct. Gene synthesis was performed by Twist Biosciences, which, using the NotI/XbaI restriction sites, also inserted the sequences into its standard vector pTwist-CMV-BetaGlobin-WPRE-Neo.

2.2 | Protein expression and purification

Using Expifectamine® (Thermo Fisher), all constructs were transfected into HEK293F cells. For in vivo biotinylation of the Avitag™ BAP, though, a second plasmid coding for BirA was co-transfected with the ACE2 vector. Upon five days of incubation in suspension, the RBD constructs were purified by IMAC using a TALON Crude column, while ACE2 was purified by immunoaffinity using a CaptureSelect XP C-tag column (Thermo Fisher). All proteins were further purified by SEC using a Superdex75 (RBD) and a Superdex200 (ACE2) column (Cytiva).

2.3 | Human sera

Human sera were obtained from 5 COVID-19 convalescent patients who were recruited at the University Hospital of Bern, Bern, Switzerland as described.³ Participants were recruited via three different routes: (a) inpatients with a SARS-CoV-2 test result (real-time PCR; RT-PCR), (b) medical personnel of the Inselspital, and (c) residual material from patients stored at the Liquid Biobank Bern (www.biobankbern.ch). Inclusion criteria of inpatients are (a) hospitalization in Inselspital, (b) tested positive for SARS-CoV-2 using RT-PCR (nasopharyngeal swab), (c) aged 18 or older, and (d) signed general consent. Human sera from 8 subjects immunized with BNT162b2 (Biontech/Pfizer) vaccine were recruited at the University Hospital of Zürich, Zürich, Switzerland.

2.4 | RBD-ACE2 binding kinetics

The binding kinetics assays were performed using an Octet RED96E (Sartorius). Briefly, SAX sensors were loaded to saturation with biotinylated ACE2 at 25 $\mu\text{g}\cdot\text{ml}^{-1}$ and subsequently quenched with biocytin. Association was carried out in 300 s, with the RBD serially diluted from 100 to 3.12 nM in 1:2 steps. Finally, dissociation was also performed in 300 s. All proteins were diluted in kinetics buffer (KB). A loaded sensor run in KB only served as drift control. The resulting curves were aligned to beginning of association, and a 1:1 model was used for global fitting.

2.5 | RBD-ACE2 binding inhibition

Inhibition of RBD-ACE2 binding by either convalescent or immunized sera was also analyzed by Biolayer Interferometry using Octet. In this case, however, HIS1K sensors were loaded to saturation with 25 $\mu\text{g}\cdot\text{mL}^{-1}$ of each RBD tested, and two association steps were performed: a short one (120 s) with the sera samples diluted at 1:20 in KB; and a longer one (300 s) with ACE2 diluted to 100 nM also in KB. Drift control was performed with a loaded sensor run in KB only. Naïve serum was used as positive control, and inhibition indexes were calculated as the proportional reduction in the area under the curve of ACE2 association.

2.6 | Anti-RBD IgG titration

For the ELISA assays, high binding 96-well plates were coated overnight with 1 $\mu\text{g}\cdot\text{mL}^{-1}$ of each of the RBDs tested; blocked for 2 h with 0.15% casein in PBS; and bound for 1 h with 1:20 convalescent or immunized sera serially diluted in 1:3 steps. Bound IgG antibodies were detected with goat anti-human IgG-POX antibody (Nordic MUBio). Development was performed with tetramethylbenzidine (TMB) and stopped with 1 M H_2SO_4 . Absorbance was read 450 nm, and the resulting curves were fitted into a 4-parameter non-linear model. OD_{50} values were then calculated as the width at half height of each fitted curve.

2.7 | Statistical analysis

Statistical tests were performed using GraphPad PRISM 9.0 (GraphPad Software, Inc.). Differences in ELISA titers and inhibition indexes were tested by non-parametric one-way ANOVA with no comparison correction (Fisher's LSD test). Statistical significance is displayed as $p \leq .05$ (*), $p \leq .01$ (**), $p \leq .001$ (***)

3 | RESULTS

3.1 | Binding kinetics of RBD mutants to ACE2 show increased affinity due L452R/E484Q and E484K mutations

To address the questions of antibody binding strength and competition mechanistically, we have expressed recombinant versions of the RBD containing mutations E484K (shared between the Brazilian isolate P.1⁸ and the South African isolate B.1.351⁹), L452R/E484Q (Indian variant B.1.617), and N440K (variant B.1.36, also emerged in India).¹⁰ The positions of these mutations relative to wild-type RBD are shown in Figure 1.

All RBDs were purified to homogeneity, and the affinity to recombinant ACE2 ectodomain was determined by BLI (Figure 2 and Table 1).¹¹ The results show that the affinity of ACE2 for

RBD_{L452R/E484Q} ($K_D = 4.6$ nM, Figure 2C) is \approx fivefold higher than that measured for RBD_{WT} ($K_D = 21.3$ nM, Figure 2A). In between are the values for RBD_{E484K} ($K_D = 19.7$ nM, Figure 2B)¹² and RBD_{N440K} ($K_D = 9.9$ nM, Figure 2D). Interestingly, in all cases, the increase in affinity is due to a strongly enhanced association rate (k_{on} , 3–5 times higher for the mutants than for RBD_{WT}), rather than any significant changes in the dissociation constant (k_{off} , Table 1).

3.2 | RBD mutants L452R/E484Q and E484K evade recognition and neutralization by convalescent sera while neutralization by BNT162b2 immune sera is impaired only

Anti-RBD IgG ELISA shows that antibodies induced by infection with wild-type Wuhan SARS-CoV-2 fail to recognize the RBD mutants L452R/E484Q (variant B.1.617) and E484K (variants P.1 and B.1.351), while recognition of RBD_{N440K} is unaltered. This observation is reproduced in the RBD-ACE2 binding inhibition assays (Figure 3B), where the neutralizing activity of the convalescent sera is significantly affected by mutations E484K and L452R/E484Q and not by N440K.

As shown previously,¹³ vaccination induces antibodies with broader specificity than viral infection, resulting in antibodies that showed reduced binding to RBD carrying mutation E484K while all other RBDs, including the Indian variants of concern were recognized well by the vaccine-induced immune sera (Figure 3C).

In contrast to direct binding of immune sera to RBD, inhibition of the RBD-ACE2 interaction was more severely affected and reduced for all mutations, most notably E484K and L452R/E484Q (Figure 3D). This most likely reflects the increased receptor affinity of the mutant RBDs for ACE2, rendering it more difficult to inhibit binding of RBD to ACE2.

4 | DISCUSSION

Recently emerged in India, the variants B.1.617 and B.1.36 of SARS-CoV-2 have rapidly spread across Southeast Asia and Northeast Africa, quickly becoming the predominant strain also in North America and Europe.¹⁴ In the UK, for example, the detection frequency of lineage B.1.617.2 has raised from $\approx 20\%$ in early May to $\approx 100\%$ in late June, which strongly indicates enhanced viral infectivity/transmissibility.

Several properties contribute to the prevalence of a particular viral strain, including its fitness and ability to escape existing immunity. Fitness is mostly influenced by viral transmissibility and replication competence, which may be affected by many factors, including viral receptor affinity. In contrast, immune evasion is normally simply driven by reduced recognition by neutralizing antibodies, with serotype formation being the most extreme form of this mechanism (whereby antibodies induced against serotype 1 of SARS-CoV completely fail to neutralize the serotype 2 of the same virus). Overall,

FIGURE 2 BLI sensorgrams illustrating the interaction between ACE2 and (A) wild-type RBD (RBD_{WT}); (B) E484K single-mutant RBD (RBD_{E484K}); (C) L452R/E484Q double-mutant RBD (RBD_{L452R/E484Q}); and (D) N440K single-mutant RBD (RBD_{N440K})

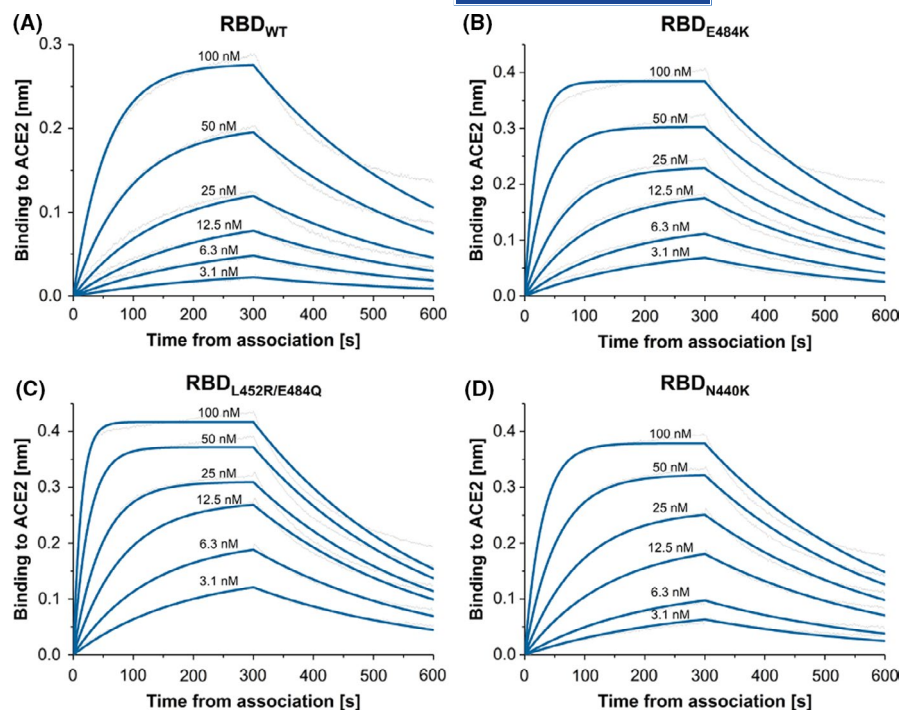


TABLE 1 Kinetic parameters for the RBD-ACE2 interaction calculated by BLI

Analyte*	K_D [M]	k_{on} [$M^{-1}s^{-1}$]	k_{off} [s^{-1}]	R^2
RBD _{WT}	21.3×10^{-9}	1.5×10^5	3.2×10^{-3}	0.9926
RBD _{E484K}	19.7×10^{-9}	1.6×10^5	3.1×10^{-3}	0.9957
RBD _{L452R/E484Q}	4.6×10^{-9}	7.2×10^5	3.3×10^{-3}	0.9934
RBD _{N440K}	9.9×10^{-9}	3.1×10^5	3.1×10^{-3}	0.9954

Note: *RBD_{WT}=Receptor-Binding Domain wild type, *RBD_{L452R/E484Q}= Receptor-Binding Domain L452R, E484Q mutations, *RBD_{E484K}= Receptor-Binding Domain E484K mutation, *RBD_{N440K}= Receptor-Binding Domain N440K mutation.

mutations on viral surface proteins involved in cell attachment and/or fusion have been postulated to be the main driver of enhanced transmissibility and/or virulence.¹⁵

Here, we assessed whether the increasing prevalence of the Indian variants of concern B.1.617 and B.1.36 could be explained by any of these 2 properties. Our data show that the affinity of RBD for ACE2 is fivefold increased by the point mutations L452R and E484Q (Figure 2C and Table 1), whereas mutation N440K has the effect of doubling this value, when compared to RBD_{WT}. In both cases though, a K-Q H-bond is likely formed, as RBD residues E484 and N440 distance by only ≈ 10 and ≈ 14 Å C $_{\alpha}$ -C $_{\alpha}$ from residues K31 and Q325 of ACE2, respectively (Figure 1). We are also tempted to speculate that mutation L452R contributes to the increased affinity observed for the double-mutant RBD mostly by stabilizing the RBM rather than by forming new bonds with the receptor. This is because L452 sits in the middle of β -sheet $\beta 5/6$, the only structured region of the RBM. Its side chain also faces the internal S349, and it seems unlikely that a drastic change in ϕ angle could occur at this position, as Y451 is deeply embedded in the internal structure of the RBD.

While these biophysical differences are truly impressive, it remains to be seen whether this increased affinity fully translates to increased viral infectivity. This is because, in oppose to what happens in vitro using soluble proteins, interaction between RBD and ACE2 in vivo is largely influenced by the environment surrounding the S glycoprotein on the virion. Nevertheless, it is reasonable to assume that significant changes in this parameter would inevitably be reflected in the dynamics between virions and host cells. This principle illustrated by the fact that the RBD of the more transmissible variant B.1.1.7 binds ACE2 with affinity twice higher than RBD_{WT}, which, in turn, interacts with the receptor four times more strongly than the RBD from the quickly controlled SARS-CoV-1.

Regarding the recognition of RBD by convalescent sera, a sharp decay is observed for mutants E484K and L452R/E484Q (Figure 3A). This result is not surprising, as mutation E484K has already been shown to evade immunity in the context of variants B.1.351 and P.1.^{12,13,16} Likewise point mutations conferred by L452R or E484Q alone have been shown to reduce sensitivity to antibodies elicited by BNT162b2 vaccine.¹⁷ Mutation N440K, on the other hand, has

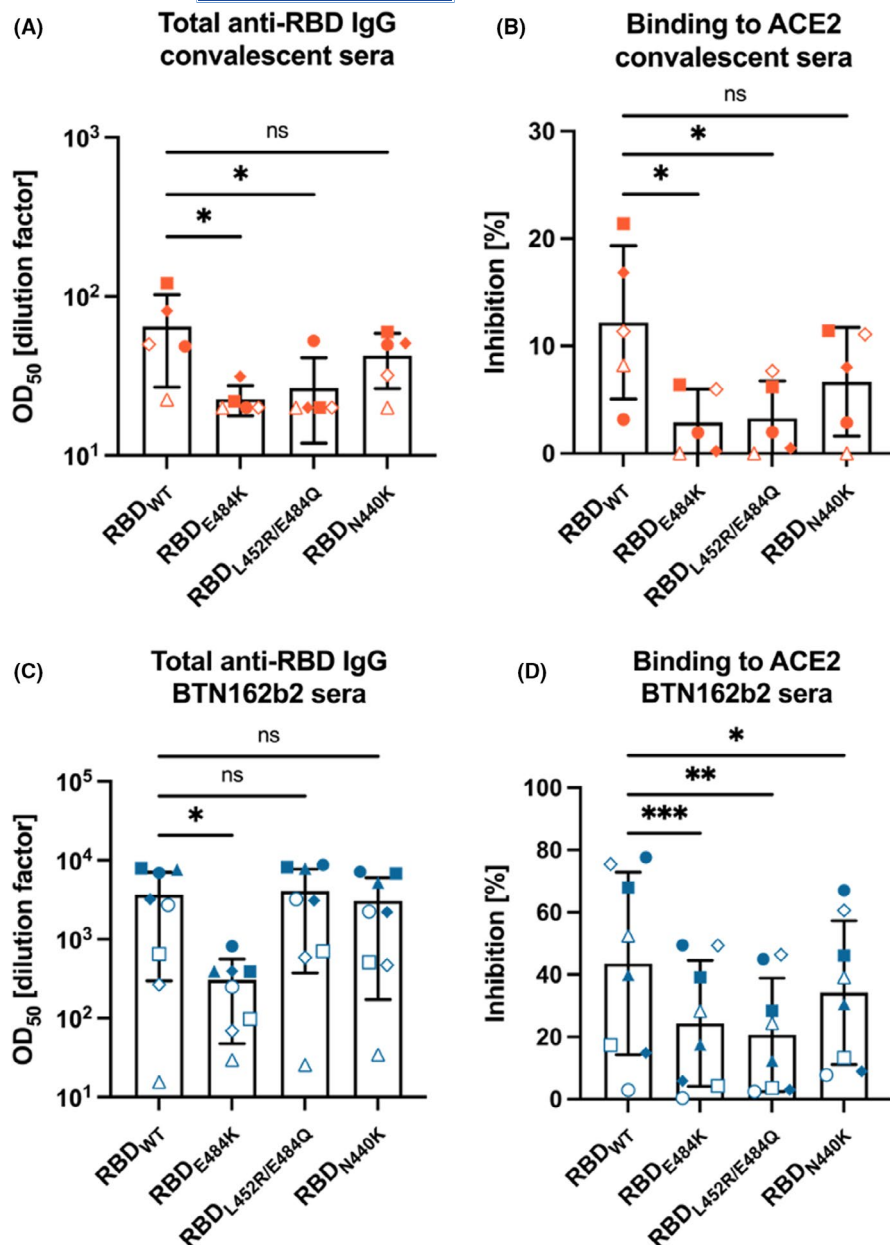


FIGURE 3 Comparison of total anti-RBD IgG and RBD-ACE2-blocking antibodies between wild-type RBD and mutants E484K, L452R/E484Q, and N440K. (A) Total anti-RBD IgG titers of sera obtained from COVID-19-convalescent donors. (B) Inhibition of RBD-ACE2 binding by 1:20 dilution of the same samples; (C) Total anti-RBD IgG titers of sera obtained from donors immunized with two doses of BNT162b2 mRNA COVID-19 vaccine (Pfizer); (D) Inhibition of RBD-ACE2 binding by 1:20 dilution of the same samples

caused no significant effect on recognition of RBD by antibodies induced by infection (Figure 3A). With the data in hands, we can only conjecture that this mutation does not alter the surface properties of the RBM sufficiently to impact its immune recognition. A similar trend appears in the results from the RBD-ACE2 binding inhibition assays (Figure 3B).

Intriguingly, the same assays performed with sera from BNT162b2-immunized donors tell a slightly different story. In this case, only mutant E484K evades recognition by anti-RBD antibodies, while all others exhibit IgG titers equivalent to those obtained for RBD_{WT} (Figure 3C). This result may be due to the change in charges (from negative to positive) that occur for E484K only, as the glutamine in E484Q is neutral at physiological pH. This might lead to a lower binding recognition of antibodies to the E484K as it has been recently postulated by free energy calculation performed

by Wu et al.¹⁸ Moreover, the overall difference in recognition patterns observed for convalescent and vaccinated sera, however, can be simply attributed to the much lower titers measured from the first group of samples, which would make slight differences more noticeable.

More interestingly though, inhibition of RBD-ACE2 interaction by vaccine-induced antibodies significantly falls for all mutants, most markedly for E484K and L452R/E484Q (Figure 3D), indicating that the increased receptor affinity renders neutralization more difficult.

Based on these results, we suggest that the RBDs of the Indian variants of concern B.1.617 and B.1.36 may have acquired enhanced infectivity/transmissibility through two distinct mechanisms: B.1.617 by increased affinity for ACE2 and immune evasion; and B.1.36 by increased affinity for ACE2 only.

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CONFLICT OF INTERESTS

M. F. Bachmann is a board member of Saiba AG, involved in the development of RBD-CuMV, a vaccine against COVID-19. All other authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

G.A., L.X. and S.K. performed and interpreted experiments. M.M. provided serum sample. M.V. and G.A. wrote the manuscript. M.B. designed experiments and wrote the manuscript.

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